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Physiological and Antigenic Characteristics of Virulent and Attenuated Strains of Legionella pneumophila (Philadelphia 3)

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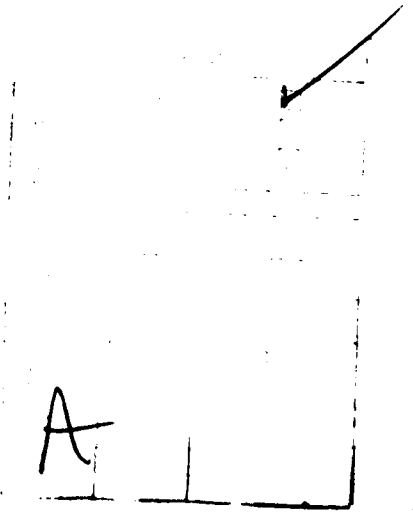

ABSTRACT

Several methods were used to characterize selected virulent and attenuated strains of Legionella pneumophila serogroup 1. The cultural characteristics of these strains on solid and liquid media were similar except that the attenuated strain grew faster. Chemical analysis revealed no significant differences between virulent and attenuated strains, nor did comparisons with three other serogroups of Legionella reveal any dissimilarities. The attenuated strain was generally more sensitive to cell wall digestion by hydrolytic enzymes. The effect of trypsin and chymotrypsin demonstrated further differences between these strains. SDS-PAGE of whole cell preparations showed only a slight difference. Antiserum directed against the virulent form showed similar microagglutination titers when either the virulent or attenuated form was used as antigen. Soluble heat extracts of the two forms, when subjected to SDS-PAGE, revealed a large 200,000 MW peptide in the attenuated strain which was absent in the virulent strain. Results of immunoelectrophoresis experiments employing adsorbed and unadsorbed antisera also suggested a difference in cellular antigens between the two strains examined.

DNA hybridization studies have been used to characterize the genus Legionella (4, 5, 8, 10, 13). The species were named after the investigators responsible for their isolation. Several species have been partially characterized and only slight differences between species have been discerned (13). Antigenic differences are demonstrated by the use of florescent antibody (16); it is by this technique that serogroups can be determined. Virulence has been observed to be a variable characteristic (15) and its regulation is unknown. It is not clear whether organisms owe their virulence to invasiveness, toxigenicity, or a combination of factors. It has been shown that Legionella multiplies within host cells and then through some combination of events causes disease. If a mechanism of virulence is related to some specific factor, either antigenic or physiologic, then studies concerning the differences existing between virulent and attenuated strains of the same species might be useful.

In this investigation we attempted to characterize partially both virulent and attenuated strains of one species of Legionella in order to elucidate, if possible, the nature of virulence.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Virulent L. pneumophila strains Philadelphia (No. 3), Togus (No. 2), Bloomington (No. 2) and Los Angeles (No. 4) and an attenuated Philadelphia (No. 3) were obtained from the Centers for Disease Control, Atlanta, Ga., on charcoal yeast extract (CYE) agar slants. Cultures were prepared from 72h CYE plates incubated at 37°C without CO_2. Broth cultures, when required, were grown as previously described (17).

Stock cultures. Virulent strains were maintained in hen yolk sac membrane homogenates stored at -70°C. Attenuated strains were obtained from a single passage from the original slants and were maintained in 0.1% tryptose in 0.85% saline stored at -70°C.

Virulence determination. Virulence was determined by a graded dose challenge using Hartley strain guinea pigs. Virulent strains gave an LD_{100} at approximately 10^5 colony-forming units (CFU), whereas the attenuated strain showed no mortality at 10^9 CFU.

Heat-extracted antigens. The antigen was prepared by suspending 6 mg of lyophilized bacteria/ml of 0.85% saline, autoclaved for 10 min at 121°C and cooled. The material was then centrifuged and the supernatant removed and used as the antigen.

Chemical analyses. Protein analysis of whole cell and cell wall suspensions was performed by the BioRad assay method (1, 2). Pentoses were determined by the occinol reaction (6). The phenol-sulfuric acid method was used to determine hexoses (9). DNA concentration was determined by the Burton assay method (6).

SDS-PAGE. Electrophoresis was performed essentially according to the method of Weber and Osborne for 7.5% acrylamide gels (19). Protein solutions (25-100 µl) were applied to 9-cm gels and electrophoresed at

a constant current of 8mA per gel column until the tracking dye was 1 cm from the bottom. The gels were stained with 0.25% buffalo black and 0.025% Coomassie brilliant blue in 10% acetic acid.

Immunoelectrophoresis. A modification of the method described by Grabar and Burton (12) was used in performing immunoelectrophoresis (IEP). Melted 1% agarose solution in IEP gel buffer (sodium barbital-barbital, I = 0.093, pH 8.6) was poured onto glass slides (24 x 74 mm). Antigen wells (0.2 cm in diameter) were cut with their centers 3 cm from the cathodic edge of the slide and 0.4 cm from the antiserum troughs, which were 0.1 x 6.5 cm. Electrophoresis was conducted at 50 mA for 60 min. The troughs were charged with antiserum and the slides were incubated at room temperature in a humid chamber for 24 h.

Cell wall preparation. The removal of the soluble cytoplasmic material was accomplished by shaking the cells with 0.10 - 0.11 mm glass beads in a Braun cell homogenizer model MSK (17). The cellular material was centrifuged so that the cell walls formed a soft coat above the pellet. This coat was removed and washed with 0.85% saline. Microscopic examination of this material revealed no whole cells.

Enzymatic digestion of the cell walls. Cell wall digestion was accomplished by a modified method of Braun and Rahn (3). Cell wall preparations were suspended at an optical density of approximately 1.0 at 573 nm then individual enzymes (Sigma) were added until a 1:50 enzyme to substrate ratio was achieved. For the measurement of the effect of different proteolytic enzymes on the absorbance of cell wall suspensions, a 0.01 M Tris solution was adjusted to pH values 8.2 (trypsin and chymotrypsin), 6.0 (papain) and 7.4 (pronase). The activation of papain was performed by incubating 5 mg of enzyme with 4.4 mg of cysteine for

1 h at room temperature in 10 ml 0.01 M Tris pH 6.0. All incubations were performed at 37°C. The incubation with lysozyme was performed using an enzyme concentration of 2.0% in 0.01 M Tris buffer pH 7.2.

Microtiter antigen. Organisms were harvested and washed once from 72-h CYE agar plates with 0.01 M phosphate buffered saline, pH 6.4. Organisms were suspended in 0.01 M PBS pH 6.4 and heated at 65°C for 1 h. The optical density of the solution was then adjusted to 0.6 at 420 nm. A small amount of sodium azide was added to prevent bacterial growth.

Microagglutination procedure. A modification of the procedure used by Farshy et al. (11) was employed for obtaining agglutinin titers for immune sera. A 1:4 dilution of the serum to be titrated was prepared using 0.01 M PBS, pH 6.4. All wells except the first row were filled with 0.05 ml PBS, then dilute serum was added to the first and second rows. Using 0.05-ml diluters, titration was initiated in the second row of wells and transferred down the full length of the plate followed by the addition to each well of 0.05 ml of the appropriate antigen. The plates were sealed to prevent evaporation then incubated for 24 h at room temperature or 48 h at 4°C.

Antiserum production. L. pneumophila of the desired strain was grown on CYE agar and harvested after 72 h. The cells were washed with 0.85% saline and brought to a final concentration of 6×10^8 CFU/ml in 0.85% saline. Dutch rabbits were injected intravenously with 1 ml of 6×10^8 CFU, once a week for three weeks. One week later blood was collected and the serum removed and stored at -70°C. Antiserum that was to be adsorbed was mixed with 1×10^{10} CFU/ml of the desired strain and incubated for 1 h at 37°C. The cells were removed by centrifugation and the serum stored at -70°C.

RESULTS

Effect of initial pH on growth. Virulent and attenuated strains of L. pneumophila Philadelphia 3 responded similarly to changes in the initial pH in a chemically defined medium. The optimal pH for both strains was 6.3 (Table 1).

Agglutination reactions. The agglutinating titers of the four serogroups and the attenuated strain of L. pneumophila Philadelphia 3 are shown in Table 2. These titers show no difference between the virulent and attenuated strains used as antigens. On the other hand, Los Angeles, Togus and Bloomington showed similarities by their cross-reactions.

Effect of enzymes. The enzymatic digestion of cell wall material is shown in Table 3. Pronase and papain were the least effective of the enzymes employed while lysozyme was moderately effective. Trypsin and chymotrypsin were active on each of the strains tested. The attenuated strain had an increased sensitivity to trypsin digestion whereas the virulent strain was more sensitive to chymotrypsin.

Chemical determinations. Analysis of the whole cell preparations of the four serogroups of L. pneumophila examined revealed similar concentrations of DNA, protein, hexose, and pentose (Table 4). Serogroup 1 had the highest values of the serogroups examined. Analysis of heat-extracted antigens from the same serogroups revealed no dissimilarities (Table 4). It can be seen that in comparison with the whole cell preparations, heating removed most of the carbohydrate and only a small amount of protein. The small amount of DNA present in these heated preparations indicates that some cellular lysis occurred during the extraction process.

SDS-PAGE. The densitometric profiles of whole cell preparations derived from the virulent and attenuated strains are compared in Fig. 1.

These profiles show that the distribution of peaks is very similar except that for the additional peak number 22 in the attenuated strain. The heat-extracted antigens derived from the virulent and attenuated strains are compared in Fig. 2. These profiles show a similarity in the distribution of peaks with the exception of peaks 1 and 2 in the attenuated strain. The peptides represented by these peaks have an approximate molecular weight of 200,000.

Immunoelectrophoresis of heat-extracted antigens. The precipitin reactions visualized after electrophoresis are seen in Fig. 3.

Homologous reactions between strain 1, heat-extracted antigens, and unadsorbed sera showed four precipitin lines and no reaction with adsorbed sera. The unadsorbed strain 1 sera gave similar reactions between heat-extracted antigens of the two biotypes of strain 3. However, adsorbed sera showed differences between virulent and attenuated strain. There is an additional antigen present in the attenuated strain that is absent from the virulent strain.

DISCUSSION

The physiologic requirements for the virulent and attenuated strains of L. pneumophila are very similar. Since it has been shown that these organisms grow intracellularly (16, 21), it was theorized that a sensitivity to low pH might be a characteristic of the attenuated strain. Our data indicates this is not the case, as the attenuated strain had the same optimal initial pH requirement as the virulent strain; however, in vitro experiments cannot duplicate the in vivo system.

Agglutination titers obtained using whole cell serogroup 1 antigens of virulent and attenuated strains and monospecific immune sera from the

other serogroups showed no cross-reactivity. On the other hand, serogroups 2-4 showed agglutinin activity among themselves. Togus, the most reactive strain tested, cross-reacted with Bloomington and Los Angeles. Bloomington reacted strongly with Togus and Los Angeles showed a one-way cross with Togus. Common antigens have been demonstrated in L. pneumophila (20); however, the distribution of agglutinin activity among these serogroups has not been clearly demonstrated. It would appear that there is more surface antigen similarity among these organisms than has previously been indicated. However, no effect on agglutinin activity following attenuation was demonstrated.

Since agglutinin activity of the virulent and attenuated strains measures cell surface antigens, it was felt that perhaps investigation of the entire cell wall would yield more conclusive data. Specific enzymatic disaggregation of the cell walls showed that the structure of the virulent and attenuated strains is different with the virulent strain containing more bonds involving carboxyl groups of aromatic amino acids. This interpretation is based indirectly on data obtained from trypsin and chymotrypsin digestion experiments. This may indicate that there is a correlation between maintenance of virulence and the requirement for specific nutritional factors. Preliminary investigations indicate this may be the case.

Chemical analyses of certain cell components showed that the attenuated strain had slightly more protein and hexose than the virulent strain, while the other three serogroups had lower concentrations. An analysis of the heat-extracted soluble components from the surface of these organisms showed no discernible differences. It appears that the differences seen with whole cell preparations represent components that

are found within the structure of cell wall or the cytoplasm of the attenuated strain. The quantity of protein extracted by the heat process was identical for both strains; however, by SDS-PAGE analysis the composition of the proteins present was shown to be different. A higher molecular weight peptide present in the attenuated strain was not found in the virulent strain.

The function of this antigen is unknown; several possible roles are proposed. This surface antigen in the attenuated strain could represent a coat or covering of the cell wall which facilitates destruction of the organism during phagocytosis. We have shown that the cell walls of the attenuated strains do contain a different structure from those of the more virulent organism. Whether this structural change is represented by a surface coat or a shift in porosity of the cell wall of the attenuated strain is unknown. It has been shown that lysis of the attenuated strain of L. pneumophila liberates a toxin; however, no extracellular toxin has been isolated from the attenuated strain (K. W. Hedlund, personal communication). This antigenic component in the attenuated strain may reduce or inactivate the toxin by binding at a specific active site.

In conclusion, differences in the virulent and attenuated strains of L. pneumophila do exist. Significant among these differences is the cell wall structure and the additional surface antigen of the attenuated strains. Further studies involving cell walls and the purification of the surface antigen in the attenuated strain could lead to elucidation of the factors involved in virulence of L. pneumophila.

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TABLE 1. Effect of initial pH on the maximum optical density at 38 h on virulent and attenuated L. pneumophila strain 3 grown in a chemically defined medium

	OD_{660}	
Initial pH	Virulent	Attenuated
5.9	0.40	0.39
6.1	0.46	0.50
6.3	0.51	0.52
6.5	0.48	0.51
6.7	0.47	0.50
6.9	0.43	0.46
7.1	0.38	0.46
7.3	0.35	0.39

TABLE 2. Agglutinin titers of immune sera with whole cells of four serogroups of L. pneumophila

Antigen	Antisera titer			
	Bloomington	Togus	L-3 Virulent[a]	Los Angeles
Bloomington	10,240	1,280	10	10
Togus	640	20,480	0	40
L-3 virulent[a]	10	40	1,280	10
L-3 attenuated[a]	0	20	2,560	0
Los Angeles	0	160	0	640

[a]Philadelphia.

TABLE 3. Effect of hydrolytic enzymes on the cell walls of virulent and attenuated L. pneumophila Philadelphia strain 3

Enzyme	Relative change in optical density[a]	
	Virulent	Attenuated
Papain	0	0.025
Pronase	0.004	0.081
Lysozyme	0.073	0.043
Trypsin	0.177	0.239
Chymotrypsin	0.191	0.031

[a]Difference in OD_{578} measured by subtracting th final OD at 50 min from the initial OD. Temperature of experimentation was 37°C.

TABLE 4. Chemical determinations of whole cell and heat-extracted preparations of L. pneumophila

Strain	µg/mg Dry weight bacteria			
	DNA	Protein	Hexose	Pentose
Whole cell				
L-3 virulent[a]	56.3 ± 11.7	817.5 ± 10.6	161.1 ± 4.1	18.2 ± 0.3
L-3 attenuated[a]	64.9 ± 1.3	863.5 ± 23.3	186.3 ± 6.0	19.7 ± 3.1
Togus	45.8 ± 1.2	593.3 ± 10.9	129.0 ± 5.9	17.8 ± 0.6
Bloomington	47.2 ± 1.7	779.3 ± 16.2	139.5 ± 10.8	14.9 ± 1.0
Los Angeles	64.1 ± 1.9	673.0 ± 21.7	128.5 ± 2.0	13.5 ± 0.5
Heat-extracted				
L-3 virulent[a]	27.7 ± 7.8	108.0 ± 4.7	115.0 ± 8.0	11.8 ± 3.1
L-3 attenuated[a]	31.4 ± 9.7	108.0 ± 8.2	115.3 ± 19.0	11.9 ± 2.5
Togus	28.5 ± 1.6	53.2 ± 1.3	113.7 ± 4.0	11.9 ± 0.4
Bloomington	25.0 ± 2.4	68.1 ± 4.7	112.0 ± 10.1	15.0 ± 0.5
Los Angeles	32.2 ± 3.8	50.3 ± 5.3	106.7 ± 7.2	14.8 ± 0.3

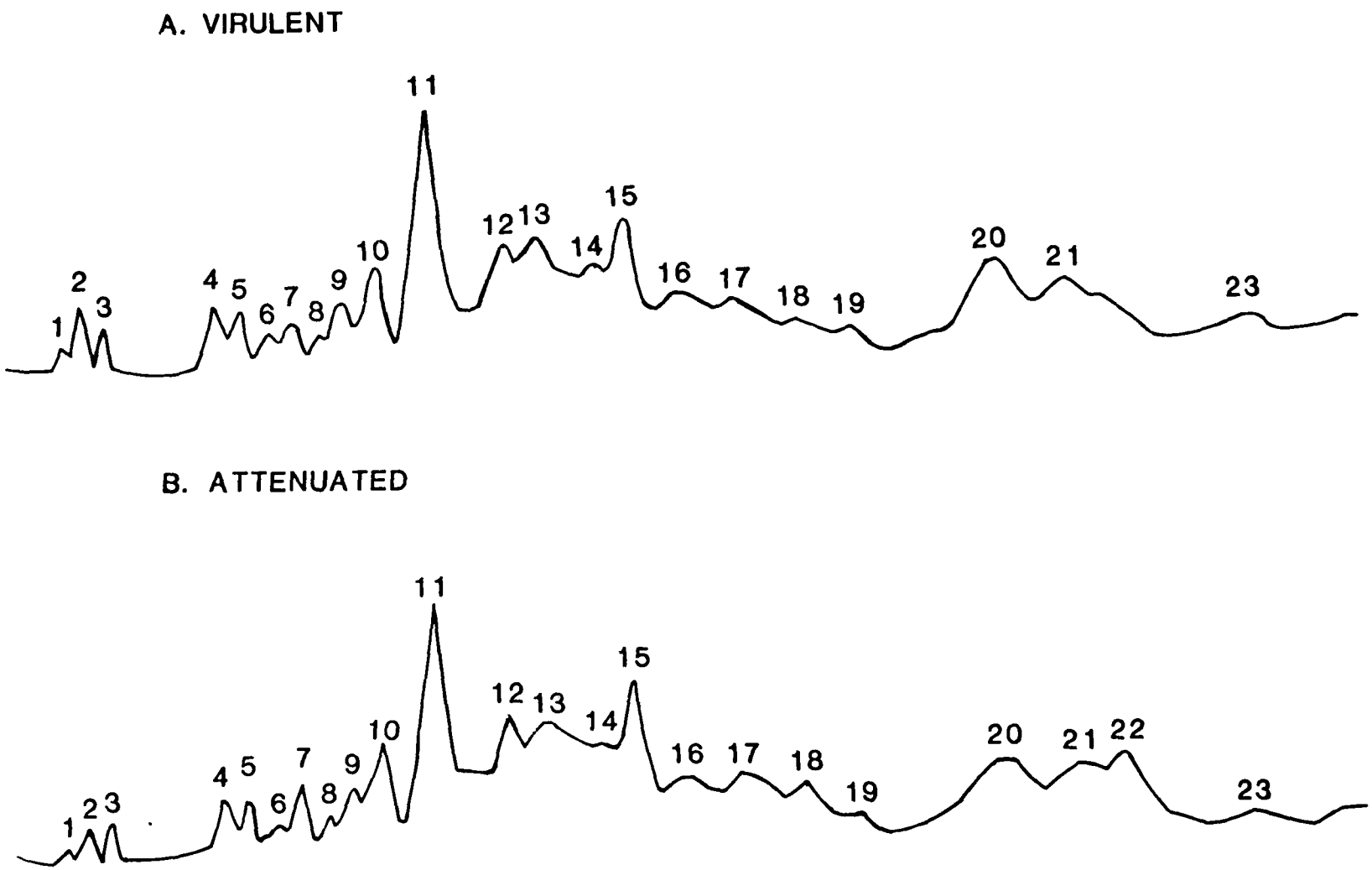
[a]Philadelphia.

FIGURE LEGENDS

FIG. 1. Typical scan of the electrophoretic protein pattern of whole cell preparations of L. pneumophila strain 3. Each protein peak is designated by a number.

FIG. 2. Typical scan of the electrophoretic protein pattern of heat-extracted antigens of L. pneumophila strain 3. Each protein peak is designated by a number.

FIG. 3. Effect of strain 1 adsorbed antiserum on immunoelectrophoresis of heat-extracted L. pneumophila antigens. Strain 1 unadsorbed antiserum vs. strain 1 antigen (A), vs. strain 3 virulent antigen (B), and vs. strain 3 attenuated antigen (D). Strain 1 adsorbed antiserum vs. strain 3 virulent (C) and vs. strain 3 attenuated antigen (E).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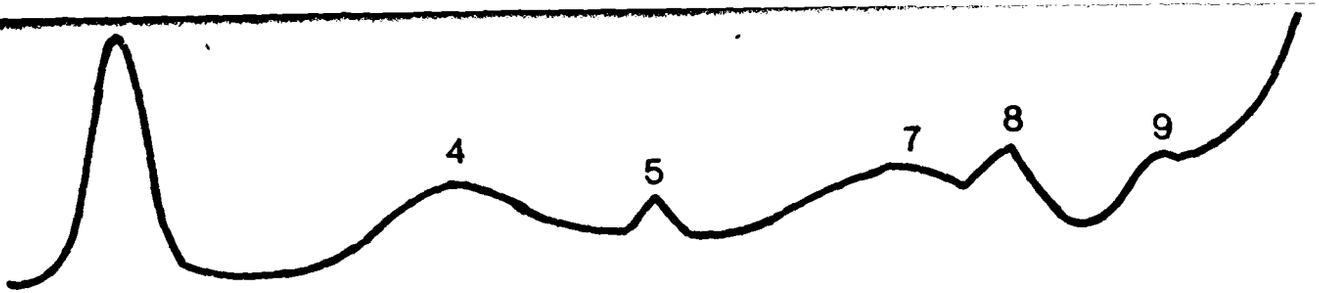

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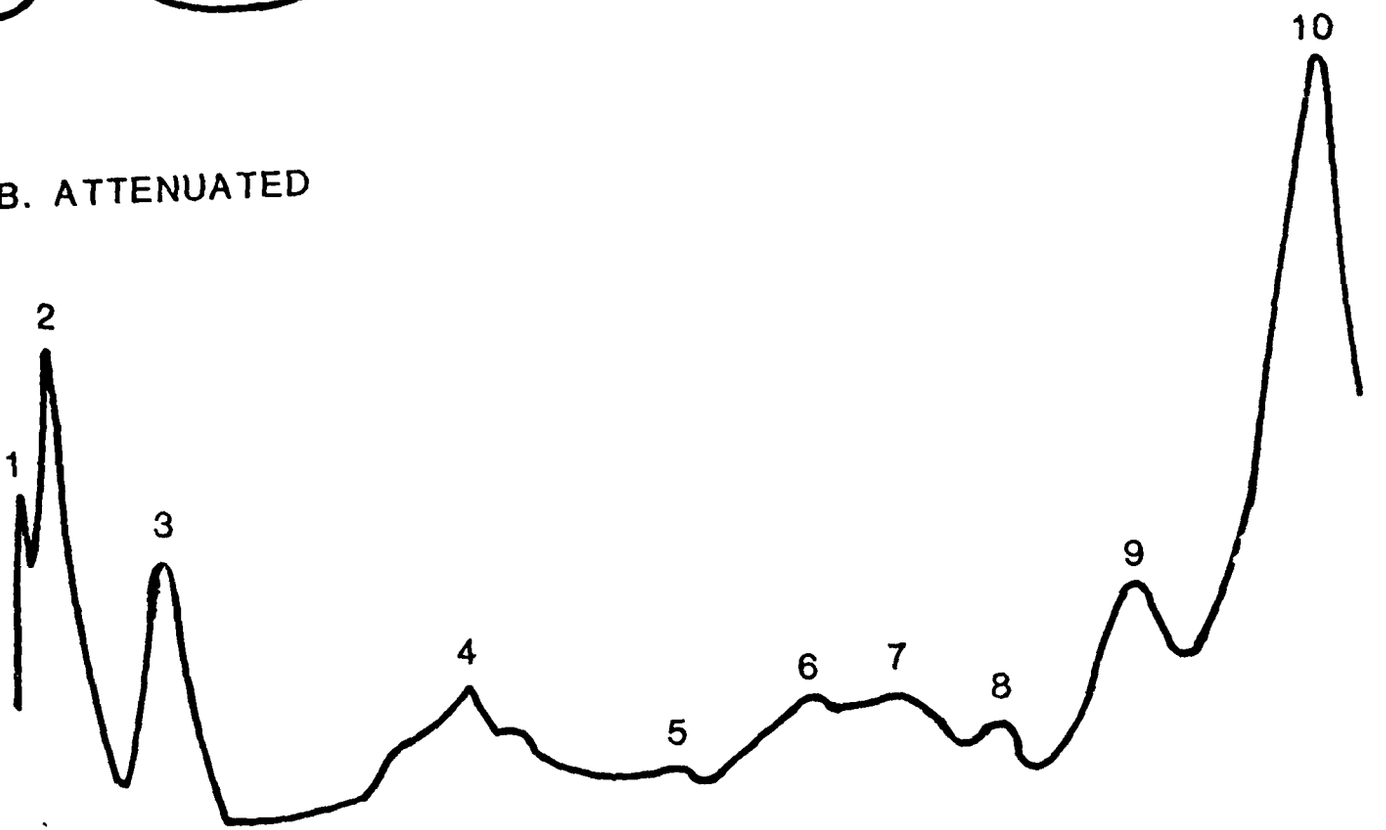


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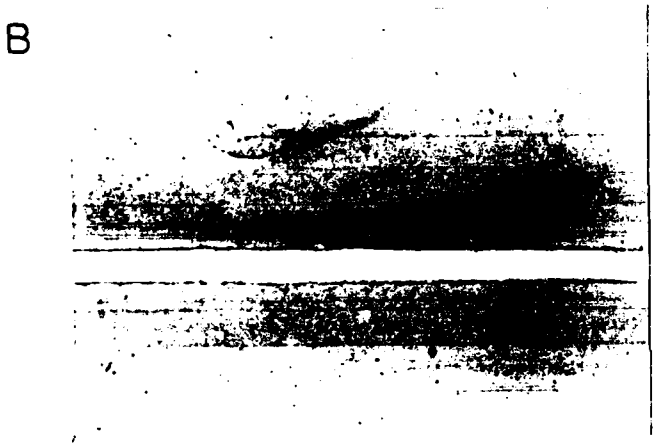
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